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(54) Title: GLYCOLATION OF GLYCOSYLATED MACROMOLECULES

(57) Abstract

A process is provided for coupling glycols to macromolecules through glycosylations on those macromolecules, rather than through amino or carboxyl groups on the macromolecule backbone. This produces macromolecules having decreased immunogenic response, and maintained activity. The present process for glycolation of a glycosylated macromolecule comprises activating a polyalkylene glycol; reacting the activated polyalkylene glycol with a diamino compound, whereby the activated polyalkylene glycol is coupled to the diamino compound through one of its amino groups; oxidizing the macromolecule to activate at least one glycosyl group therein; and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule. The result is a glycolated glycosylated macromolecule, wherein a glycol is bonded to the macromolecule through its glycosylations.

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GLYCOLATION OF GLYCOSYLATED MACROMOLECULES

Background of the Invention

The use of polypeptides in circulatory systems for the purpose of engendering a particular physiological response is well known in the medicinal arts. Among the best known polypeptides utilized for this purpose is insulin, which is used in the treatment of diabetes. Another group of polypeptides to which great therapeutic potential has been attributed are various enzymes. A principal factor which has severely limited the use in therapeutics of polypeptides is that most of these compounds elicit an immunogenic response in body fluids, evidenced by changes in the composition of the circulatory system, i.e., the production of antibodies to the polypeptides. This effect has one or both of two secondary consequences: first, neutralization of the polypeptides by the antibodies thus produced; second, and more seriously, the development of an allergic response.

Neutralization of polypeptides by antibodies is believed to be responsible for the rather low residence time of insulin in the human circulatory system; hence, persons afflicted with diabetes are forced to inject themselves fairly frequently with fresh doses of insulin. In the case of parenterally administered enzymes, not only is there the problem of neutralization of the polypeptide and the subsequent negation of its physiological activity, but also the extremely undesirable elicitation of an allergic reaction.

Overall, a limitation to the potential therapeutic benefit derived from the clinical use of polypeptides is

their potential for eliciting such immune response in the circulatory system. This immune response may be caused by aggregates in the material prior to injection as described by R. Illig (1970), J. Clin. Endocr., 31, 679-688, W. Moore (178), J. Clin. Endrocrinol. Metab., 51, 691-697. The antibody production may decrease or eliminate the desired biological function of the polypeptide, sometimes by causing reduced residence time in the circulatory system (reduced half-life) or by modifying the molecule by virtue of the antibody-polypeptide interaction.

The problems set forth hereinabove are well recognized, and various approaches have been taken in attempts to solve them. The attachment of enzymes to insoluble supports has been the subject of a great deal of work. Reviews dealing with this subject will be found in Silman and Katchalski, Ann. Rev. Biochem., 35, 387 (1966), and Goldstein, Fermentation Advances, Academic Press, New York (1969), page 391. This approach, however, while of academic interest, does not provide injectable long-life polypeptides. Another approach which has been taken to provide polypeptides of lengthened in vivo life has been the microencapsulation of enzymes which has been discussed in numerous articles by Chang and coworkers, namely, Science, 146, 524 (1964); Trans. Am. Soc., 12, 13 (1966); Nature, 218, 243 (1968); Can. J. Physiol. Pharmacol., 45, 705 (1967). A further approach has been the heat stabilization of enzymes by attaching carboxy methylcellulose to an enzyme such as Trypsin (Mitz and Summaria, Nature, 198, 576 (1961), and the attachment of proteases to hydrophilic carriers (Brummer et al., Eur. J. Biochem., 25, 129 (1972). These approaches, however, do not provide polypeptides in a soluble form, which form is the most desirable for injection and dosage control of injectable materials. Yet a further approach has been the attachment of synthetic polymers to polypeptidal proteins. A review of this work is found in Sela, "Advances in Immunology," 5, 30 (1966), Academic Press,

New York. In this work, it has been shown that while homopolymers of amino acids are nearly all non-immunogenic, when these polymers are attached to immunogenic proteins, the immunogenic activity is not masked, and antibodies are produced in test circulatory systems. For example, while polyglycine itself is non-immunogenic, when attached to a protein, that conjugated protein becomes a hapten. Similarly, while dextran itself is slightly immunogenic, when coupled to insulin, the insulin-dextran-coupled material is believed to become substantially immunogenic.

Other modifications of polypeptides include the modification of proteins with substantially straight-chain polymers such as polyethylene (PEG) or polypropylene glycol (PPG).

For example, U.S. Patent No. 4,055,635 discloses pharmaceutical compositions comprising a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as polysaccharides.

U.S. Patent No. 4,088,538 discloses a reversibly soluble, enzymatically active polymer enzyme product comprising an enzyme covalently bonded to an organic polymer such as polyethylene glycol.

U.S. Patent No. 4,415,665 discloses a method of conjugating an organic ligand containing at least one primary or secondary amino group, at least one thiol group, and/or at least one aromatic hydroxy group (described in column 3, lines 19-36) to a polymeric carrier with at least one hydroxyl group (described in column 2, lines 42-66).

U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1-proteinase inhibitor with a polymer such as PEG or methoxypolyethylene glycols.

Abuchowski et al., J. Biol. Chem. 252(11), p. 3578, disclose covalent attachment to an amino group of bovine serum albumin of methoxypolyethylene glycols.

U.S. Patent No. 3,619,371 discloses a polymeric matrix having a biologically active substance chemically bound thereto.

U.S. Patent No. 3,788,948 discloses use of organic cyanate compounds to bind proteins to polymers.

U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a proteolytic enzyme linked covalently to a polymeric substance.

JP 57-92435, published November 26, 1982, discloses modified polypeptides, where all or part of the amino groups are substituted with a polyethoxyl moiety. DE 2312615, published September 27, 1973, discloses conjugating of polymers to compounds containing hydroxy or amino groups.

EP 147,761 discloses a covalent conjugate of α-1-proteinase inhibitor and a water-soluble polymer, where the polymer may be polyethylene glycol.

EP 154,316, published September 11, 1985, discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of a lymphokine.

U.S. Patent No. 4,414,147 describes rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride).

PCT WO 87/00056, published January 15, 1987, discloses conjugation of PEG and polyoxyethylated polyols to such proteins as interferon-β, interleukin-2, and immunotoxins.

Davis et al., U.S. Patent No. 4,179,337, provide peptides and polypeptides coupled to polymers which are substantially non-immunogenic. In the process of Davis et al., a substantially straight-chain polymer is modified, suitably at one end thereof, either by the alteration of the terminal group or by the addition thereto of a coupling group having activity vis-a-vis polypeptide and reacting said activated polymer with the polypeptide.

Davis et al. indicate that the glycol couples most likely through an amino group on the protein, but also discloses an embodiment where the terminal hydroxy group of the glycol is converted to an amino group, e.g., with a sulfonating agent or a halogenating agent, and the resultant halide or tosylate is coupled with a carboxyl group of the polypeptide by known methods. Although the reduction of biological activity is less than where amino groups on the protein are the coupling sides, the activity is still reduced.

Summary of the Invention

It is an object of the present invention to provide a method for reducing the immunogenicity of biologically active macromolecules, while maintaining their activity.

Upon further study of the specification and appended claims, further objects and advantages of this invention will become apparent to those skilled in the art.

These objects have been satisfied by providing a process for coupling glycols to macromolecules through glycosylations on those macromolecules, instead of through amino or carboxyl groups on the macromolecule backbone itself, and by providing macromolecules having unexpectedly decreased immunogenic response and surprisingly maintained activity. Molecules in accordance with the invention, in addition, exhibit increased biological half-life, due to steric blocking of clearance receptors; increased solubility of hydrophobic molecules in an aqueous environment, due to the addition of lipophilic moieties; and increased resistance to proteolysis, due to steric hindrance.

One aspect of the invention, therefore, is a process for the glycolation of a glycosylated macromolecule, comprising activating a polyalkylene glycol, reacting the activated polyalkylene glycol with a diamino compound, whereby the activated polyalkylene glycol is coupled to the diamino compound through one of its amino groups,

oxidizing the macromolecule to activate at least one glycosyl group therein, and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule. Specifically, the invention preferably comprises a process for the PEGylation of a glycosylated macromolecule comprising:

(a) reacting a polyethylene glycol of the formula $CH_3O-(CH_2CH_2O)_n-H$ with o-nitrophenylchloroformate and triethylamine to produce a nitro compound of the formula $CH_3O-(CH_2CH_2O)_n-COO-Ph-NO_2$,

(b) reacting the nitro compound with a diaminoalkane of the formula $H_2N-(CH_2)_x-NH_2$ to produce an amino compound of the formula $CH_3O-(CH_2CH_2O)_n-CO-NH-(CH_2)_x-NH_2$,

(c) oxidizing sugar groups on the macromolecule to produce a macromolecule with an oxidized sugar residue, and

(d) reacting the amino compound with the activated macromolecule to produce a PEGylated molecule. The preferred molecular weight of the polyethylene glycol is up to about 24,000; accordingly, n is preferably about 2-500. In the diaminoalkane, x is preferably about 1-20.

The result of this preferred process is a PEGylated glycosylated macromolecule, wherein PEG is bonded to the macromolecule through its glycosylations, specifically, of the formula PEG-OCO-NH-alkylene-N=CH-macromolecule.

Macromolecules usable in the invention include virtually any bioactive macromolecule bearing glycosylations (regardless of how bonded, e.g., covalently, etc.) or which can be glycosylated, e.g., polypeptides and/or proteins, nucleic acids, lipids, or carbohydrates. Preferred peptides include those comprising an antigen binding region, a cytokine, a receptor, an antithrombotic, a growth factor, or an angiohypotensive reagent. More preferably, the polypeptide is an immunoglobulin, an interferon, a receptor tyrosine kinase, a thrombomodulin, a transforming growth factor, an endothelin, or an analog of the above. One especially preferred protein is the

monoclonal antibody TAb-250 or its chimeric analog BACh 250 ("BACh-250" or "C-erb-B2"). See Molecular Oncology as a Basis for New Strateties in Cancer Therapy: Efficacy of an Anti-c-erbB-2 Mouse/Human Chimeric Antibody Alone and in Combination with cis-Diammedichloroplatinum (CDDP), Langton et al., Proceedings of the 2nd Joint Meeting of the American Association for Cancer Research and the Japenese Cancer Association.

The procedures of the present invention are applicable to enzymes and peptide hormones. Examples of enzymes which can be used are:

oxidoreductases, such as Urate:oxygen-oxidoreductase, Hydrogen-peroxide:hydrogen-peroxide oxidoreductase, Cholesterol-reduced-NADP:oxygen oxidoreductase (20-β-hydroxylating);

transferases, such as UDP glucuronate glucuronyl-transferase (acceptor unspecific), UDP glucose:α-D-Galactose-1-phosphate;

hydrolases, such as Mucopeptide N-acetyl-muramyl-hydrolase, Trypsin, L-asparagine aminohydrolase;

lyases, such as Fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase;

isomerases, such as D-Xylose ketol-isomerase; and

ligases, such as L-Citrulline and L-aspartate ligase.

Examples of peptide hormones that can be used are insulin, ACTH, Glycagon, Somatostatin, Somatotropin, Thymosis, Parathyroid hormone, Pigmentary hormones, Somatomedin, Erythropoietin, Luteinizing hormone, Chorionic Conadotropin, Hypothalmic-releasing factors, Antidiuretic hormones, Thyroid-stimulating hormone, and Prolactin.

Macromolecules which have no sugars may be glycosylated by means which are well known in the art, e.g., as disclosed in Creighton, Proteins, W.H. Freeman & Co., New

York, 1983. Virtually any sugar which is reactive to oxidation is suitable. Examples include galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, sialic acids, fucose, and/or xylose. The length of the carbohydrate chains of the sugar may vary widely, i.e., poly- and oligosaccharides may be used. Normally, the glycosylation will be that which is indigenous to the species producing the macromolecule, e.g., mammalian.

The glycol is preferably polyethylene glycol, polypropylene glycol, or a mixture thereof, as well as a mixed polyethylene-polypropylene glycol. Polyethylene glycols (PEG's) are most preferred, especially monomethoxyethylene glycol. Preferred polyethylene glycols have the formula $CH_3O-(CH_2CH_2O)_n-H$, wherein n is 2-500, more preferably 20-400.

The diamino compound preferably has the formula $H_2N-R-NH_2$, wherein R is an organic moiety. R may be, for example, a C_{1-20}-aliphatic or C_{3-20}-cycloaliphatic moiety or a C_{5-20}-aryl moiety. Aliphatic moieties include straight- or branched-chain or cyclic alkyl, alkenyl, dienyl, and alkynyl groups. Preferred aliphatic moieties are C_{2-12}-alkyl groups. Preferred aryl moieties are heterocyclic, i.e., containing one or more O, S, or N atoms, and aromatic, e.g., phenyl groups.

Activation of the glycol with the addition of the diamino compound preferably occurs as in Veronese et al., Applied Biochemistry-Biotechnology, Vol. 11, pp. 141-152 (1985). The glycol is activated by reaction with 2,4,5-trichlorophenyl-chloroformate or p-nitrophenylchloroformate and triethylamine to yield a glycolphenylcarbonate, which is then reacted with the diamino compound in high excess so that only one amino group of the diamino compound reacts with the activated glycol. Preferably, the molar ratio of diamino compound to activated glycol is at least 2:1, and more preferably at least 10:1. The reaction proceeds rapidly for a time preferably from about 1 minute to 2 hours, more preferably from

about 5 minutes to an hour. Typically temperatures from 4°C to 100°C will be utilized, preferably between 10° and 60°, more preferably near ambient, i.e., room temperature.

Alternatively, the glycol may be activated as in Davis et al., U.S. Patent No. 4,179,337, by reacting the glycol at its terminal hydroxyl group either with a sulfonating agent, such as toluene chloride, or with a halogenating agent, such as triphenyl phosphine in carbon tetrachloride or triphenyl phosphine with a suitable N-halosuccimide. The thus-produced halide or tosylate is then treated with sodium azide and reduced with lithium aluminum hydride to give the corresponding terminal amino compound.

The activated, amino group-containing glycol is then reacted with the macromolecule, on which a carbohydrate, i.e., a sugar moiety, has been oxidized. Preferably, oxidation is accomplished by reaction of the macromolecule with sodium periodate ($NaIO_4$) in a preferred molar ratio of sugar to $NaIO_4$ of 1000:1 to 10:1. The concentration of $NaIO_4$ is preferably 10-1000 mM. The reaction may preferably proceed at temperatures of 0°C to 50°C, and preferably for 1 minute to 4 hours, more preferably about 30 minutes to 2 hours, most preferably about 30 minutes. Adjustment of the reaction time may be made to control the amount of glycols per protein, since longer incubation results in greater oxidation of the sugars and, accordingly, more points on the macromolecule available for glycol attachment. Care should be taken to avoid over-glycolation, resulting in stearic hinderance of the macromolecule, reducing efficient reaction with its intended target. One of ordinary skill in the art could easily, with only routine experimentation, optimize this portion of the process for an intended use of the macromolecule.

Following oxidation of the sugars, the macromolecule is coupled with the amino group-bearing glycol, by simple

mixing, at temperatures preferably of about 4°C to 100°C, and for times preferably from about 1 minute to 5 hours, more typically between 3 minutes and 1 hour, and more preferably between 5 minutes and 30 minutes. Isolation, if desired, and work-up for biological applications is conventional, as disclosed in, e.g., U.S. Patent No. 4,179,337 or as described below. The macromolecules may be used as diagnostic reagents, therapeutic reagents, test samples, etc., as known in the art and dependent on their disclosed biological utilities.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative and not limitative of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

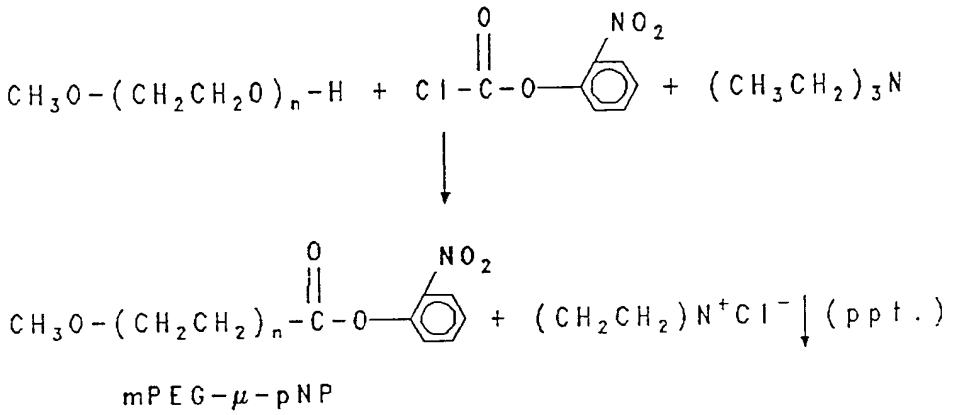
The entire disclosure of all applications, patents and publications, cited above and below, are hereby incorporated by reference.

EXAMPLES

1. Activation of Methoxy-PEG (mPEG)

Two grams of mPEG (0.1 mM, final concentration), 15 Kd, is dissolved in 20 ml of acetonitrile with 0.24 g of o-nitrophenylchloroformate (1.2 mM) and 33 µl of triethylamine (1.2 mM) and stirred for 24 hours at room temperature.

Chemical Reaction



mPEG-µ-pNP

The triethylammonium chloride is then filtered off using a sintered glass funnel. 200 ml of ethyl ether is added, and the solution is left to crystallize overnight at 4°C. The product is filtered, washed with ether to remove all of the yellow color, and recrystallized from acetonitrile-ether. The yield is 75%. The product is then assayed spectrophotometrically by the release of p-nitrophenol by ϵ-amino-n-caproic acid (ACA).

The purity of the product is further verified spectrophotometrically.

2. PEGylation of TAb-250 Through Lysine Groups

Protocol

5 mg of TAb-250 (0.0316 µmoles) is dialyzed extensively into 50 mM sodium borate buffer pH 8.3. A lower pH is used in order to ensure that only the very reactive epsilon amino groups of lysine are PEGylated.

To the 2 ml dialyzed sample 3 mg of the activated mPEG is added, a 5 molar excess. Every 30 minutes, 5 μl is removed and mixed with 5 μl of 25 mM ACA. Immediately, 3 mg of activated mPEG is added and incubated at room temperature with shaking for a further 30 minutes. The reaction is stopped after 2 hours; final molar excess is 20-fold, by loading the sample on a NAP 25 (Pharmacia) desalting column and eluting it with 50 mM $NaPO_4$ buffer, pH 6.8. The desalted sample is loaded on Superose 6 column (1 x 30 cm BioRad Econocolumn®) and eluted with 50 mM $NaPO_4$ buffer, pH 6.8. Four resultant peaks from the Superose column and the 30-minute time point samples are assayed by SDS-PAGE. The Superose peaks are further assayed by Radial Immuno-diffusion (RID) from Tago Immuno, Inc. for the quantitation of mouse IgG_1, more specifically a kappa light chain. See Table 1.

Half of the pooled superose b sample is PEGylated as described above; however, this time, activated PEG was in 100x molar excess and incubated for 3 hours. Half of this sample is allowed to incubate overnight before being stopped.

All samples were processed and assayed as reported above.

Chemistry:

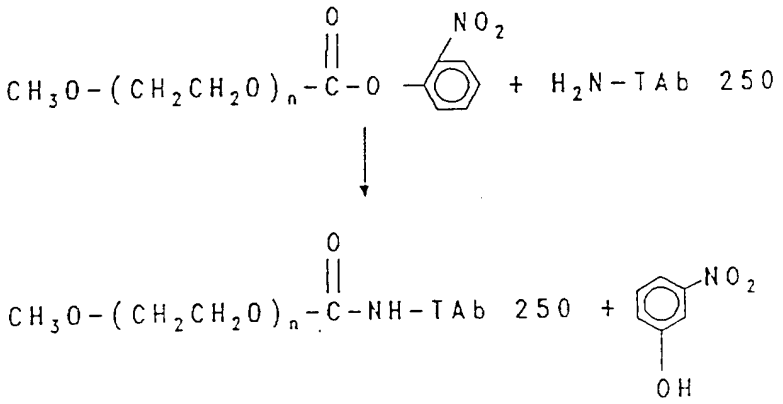


TABLE 1

Sample	Diameter	µg/mL	Est. Binding Efficiency %
PEG-µ-butamine TAb 250	11.6	Out of range	100
PEG-µ-TAb 250 100x excess	5.6	25	16.7
TAb 250 in borate pH 8.3	6.8	150	100
PEG-µ-TAb 250 20x (peaks 1+2)	7.7	129	100
Oxidized TAb 250	5.3	51.3	100
Tago Standards IgG Tago Immuno, Inc.	5.5	0.16/mg/mL (Conc. on vial = 0.156 mg/ml)	

3. **PEGylation of TAb-250 Through the Carbohydrate Moieties**

Immunoglobulin G (IgG_1) contains approximately 3% carbohydrate by weight linked to the F_c region of the protein.

Protocols

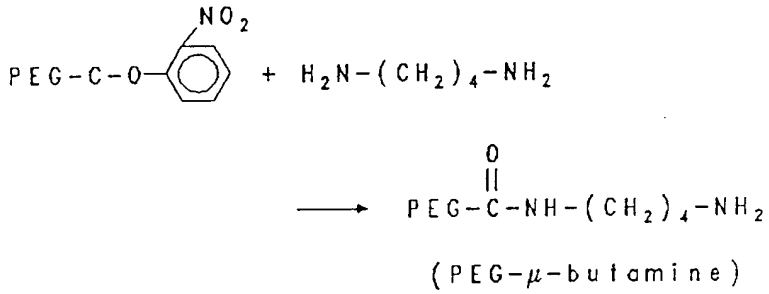
(a) Making the amino derivative of mPEG-µ-pNP

0.5 g of mPEG-µ-p-nitrophenyl is slowly added to 5 mL of 50 mM Na-borate buffer, pH 9.0, containing 44.25 mg (100 mmoles) of 1,4-aminobutane. The reaction is incubated at room temperature with shaking for 3 hours. The reaction is stopped by passing it through an NAP 25 desalting column and eluted with water and dialyzed into milli-Q H_2O.

The dialyzed material is lypophylized and weighed.

Total yield of PEG-µ-butamine = 0.573 g.

Chemistry



During the reaction, 1,4-diaminobutane is in high excess to guard against reacting both amino groups.

(b) Oxidation of TAb-250

Immunoglobulin G_1 (IgG_1) contains approximately 3% carbohydrate by weight linked to the F_c region of the protein.

Coupling Buffer:	0.05 M sodium acetate
	0.1 M sodium chloride, pH 5.0
Wash Buffer:	0.1 M sodium acetate
	0.5 M sodium chloride, pH 3.5
Storage buffer:	0.05 M sodium phosphate, pH 6.8

0.5 mg of TAb-250 is buffer exchanged into the coupling buffer using an NAP-10 (Pharmacia) desalting column. To the TAb-250 solution is added 0.1 mL of freshly prepared 100 mM sodium m-periodate ($NaIO_4$). The solution is mixed gently, and the sealed reaction vial is shielded from light and incubated at room temperature for 30 minutes. To stop the reaction, the sample is passed through a NAP-10 desalting column and is equilibrated with wash buffer. The column is eluted with the conjugation buffer.

(c) Coupling of oxidized TAb-250 to PEG-μ-butamine

To the desalted, oxidized TAb-250 is added 5 mg of PEG-μ-butamine. The reaction vial is overlayed with nitrogen and is tumbled gently overnight at 4°C. The

molar ratio of TAb-250 to PEG-μ-butamine is 1:100. The sample is then loaded following optional reduction of the TAb-250 onto the same Superose 6 column. The IgG peaks are pooled and are concentrated on an amicon stirred cell concentrator.

All the pegylated TAb 250 species were further analyzed by SDS-PAGE so as to estimate their molecular weights.

Discussion

From the SDS-PAGE gel analysis, it can be seen that the experiments have generated species of a higher molecular weight than TAb-250. Table 1 demonstrates that 100x molar excess sample has lost most of its binding capacity, as expected. It would indicate that all of the lysine binding sites are saturated, thus hindering binding. The 20x molar PEG excess and the carbohydrate conjugated samples all appear to retain all of their binding capacity.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. A glycolated, glycosylated macromolecule, wherein a glycol is bonded to the macromolecule through a glycosylation moiety.

2. A glycolated, glycosylated macromolecule according to claim 1, wherein the glycol is a polyalkylene glycol.

3. A glycolated, glycosylated macromolecule according to claim 2, wherein the glycol is a polyethylene glycol.

4. A glycolated, glycosylated macromolecule according to claim 1 of the formula:

glycol-diamine-macromolecule,

wherein the diamine is bonded to the macromolecule through a carbohydrate moiety thereof, thereby forming a Schiff base linkage.

5. A glycolated, glycosylated macromolecule according to claim 1 of the formula:

glycol-OCO-NH-alkylene-N=CH-macromolecule,

wherein "alkylene" has 1 to 20 carbon atoms.

6. A glycolated, glycosylated macromolecule according to claim 1, which is a pharmacologically active compound.

7. A glycolated, glycosylated macromolecule according to claim 1, wherein said macromolecule comprises a nucleic acid, a polypeptide, or a lipid.

8. A glycolated, glycosylated macromolecule according to claim 7, wherein the polypeptide is a protein.

9. A glycolated, glycosylated macromolecule according to claim 8, wherein the protein is TAb-250 or BACh-250.

10. A glycolated, glycosylated macromolecule according to claim 1, having essentially undiminished bioactive half-life in a host and a reduced immunogenic side effect, an increased aqueous solubility, an increased resistance to proteolytic digestion, or a decreased affinity for formulation polymers, as compared to said glycosylated macromolecule which is not glycolated.

11. A glycolated, glycosylated macromolecule according to claim 1, having increased bioactive half-life compared to a glycosylated macromolecule which is not glycolated.

12. A glycolated, glycosylated macromolecule according to claim 1, having essentially undiminished bioactive half-life in a host, as compared to said glycosylated macromolecule which is glycolated through amino or carboxyl groups not on the glycosyl portion of the macromolecule.

13. A glycolated, glycosylated macromolecule according to claim 7, wherein said macromolecule is a polypeptide which comprises an antigen binding region.

14. A glycolated, glycosylated macromolecule according to claim 7, wherein said macromolecule is a polypeptide which is a cytokine, a receptor, an anti-thrombotic, a growth factor, or an angiohypotensive reagent.

15. A glycolated, glycosylated macromolecule according to claim 7, wherein said polypeptide is an immunoglobulin, an interferon, a receptor tyrosine kinase, a thrombomodulin, a transforming growth factor, or an endothelin.

16. A process for the reduction of immunogenic side effect, for increasing aqueous solubility, for increasing resistance to proteolytic digestion, or for increasing the half-life of a bioactive glycosylated macromolecule upon administration to a host, comprising glycolating said macromolecule by bonding glycol to a carbohydrate moiety thereof.

17. A process according to claim 16, wherein the glycol is bonded to the macromolecule by a Schiff base linkage.

18. A process according to claim 16, wherein the macromolecule is a polypeptide.

19. A process according to claim 18, wherein the polypeptide is a protein.

20. A process according to claim 19, wherein the protein is TAb-250 or BACh-250.

21. A process for the glycolation of a glycosylated macromolecule, comprising activating a polyalkylene glycol, reacting the activated polyalkylene glycol with a diamino compound whereby the activated polyalkylene

glycol is coupled to the diamino compound through one of its amino groups, oxidizing the macromolecule to activate at least one glycosyl group therein, and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule.

22. A method of claim 21, wherein said activating of polyalkylene glycol produces a glycol-nitrophenyl carbonate intermediate.

23. A process according to claim 21, wherein said diamino compound has the formula $H_2N-R-NH_2$, wherein R is an organic moiety.

24. A process according to claim 23, wherein R is an aliphatic hydrocarbon having from 2 to 20 carbon atoms or an aromatic hydrocarbon having from 5 to 20 carbon atoms.

25. A process according to claim 24, wherein R is an aliphatic hydrocarbon having from 5-12 carbon atoms.

26. A process according to claim 21 for the PEGylation of a glycosylated macromolecule comprising:

(a) reacting a polyethylene glycol of the formula

$$CH_3O-(CH_2CH_2O)_n-H$$

with o-nitrophenylchloroformate and triethylamine to produce a nitro compound of the formula

$$CH_3O-(CH_2CH_2O)_n-COO-Ph-NO_2,$$

(b) reacting the nitro compound with a diaminoalkane of the formula

$$H_2N-(CH_2)_x-NH_2$$

to produce an amino compound of the formula

$$CH_3O-(CH_2CH_2O)_n-CO-NH-(CH_2)_x-NH_2,$$

(c) oxidizing sugar groups on the macromolecule to produce a macromolecule with an oxidized sugar residue, and

(d) reacting the amino compound with the macromolecule to produce a PEGylated molecule, wherein n is 2-500 and x is 1-20.

27. A process according to claim 26, wherein n is 20-400 and x is 2-12.

28. A process according to claim 21, wherein the macromolecule is a nucleic acid, a polypeptide, or a lipid.

29. A process according to claim 21, wherein the macromolecule is a pharmacologically active compound.

30. A glycolated, glycosylated macromolecule produced by a process of claim 16.

31. A glycolated, glycosylated macromolecule produced by a process of claim 21.

32. A PEGylated, glycosylated macromolecule produced by a process of claim 26.

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(57) Abstract

A process is provided for coupling glycols to macromolecules through glycosylations on those macromolecules, rather than through amino or carboxyl groups on the macromolecule backbone. This produces macromolecules having decreased immunogenic response, and maintained activity. The present process for glycolation of a glycosylated macromolecule comprises activating a polyalkylene glycol; reacting the activated polyalkylene glycol with a diamino compound, whereby the activated polyalkylene glycol is coupled to the diamino compound through one of its amino groups; oxidizing the macromolecule to activate at least one glycosyl group therein; and reacting the polyalkylene glycol coupled to the diamino compound with the oxidized glycosyl group in the macromolecule. The result is a glycolated glycosylated macromolecule, wherein a glycol is bonded to the macromolecule through its glycosylations.

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Internati Application No
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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	CHEMICAL ABSTRACTS, vol. 103, no. 11, 16 September 1985, Columbus, Ohio, US; abstract no. 84021, cited in the application see abstract & APPL. BIOCHEM. BIOTECHNOL., vol.11, no.2, 1985 pages 141 - 152 VERONESE F. ET AL. 'SURFACE MOFIFICATION OF PROTEINS. ACTIVATION OF MONOMETHOXY-POLYETHYLENE GLYCOLS BY PHENYLCHLOROFORMATES AND MODIFICATION OF RIBONUCLEASE AND SUPEROXIDE DISMUTASE.' --- -/--	1-32

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio Application No
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